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Radioimmunoassay of Nortestosterone and Related Steroids

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Summary: A radioimmunoassay of nortestosterone and related steroids, including its principal metabolites, is described and evaluated. Antisera against nortestosterone-17β-hemisuccinate- and nortestosterone-3-carboxymethyloxime-bovine serum albumin were raised in goats. By using a mixture of such antisera with different selectivity, the cross-reactions of several naturally occurring steroids can be reduced. The method can be applied for the detection of nortestosterone in both unprocessed or hydrolyzed urine extracts and also in plasma. It has been used as a screening test for anabolics in doping control.

Radioimmunoassay für Nortestosteron und verwandte Steroide

Zusammenfassung: Für die Bestimmung von Nortestosteron, seine Metabolite und verwandte synthetische anabole Steroide wurde ein Radioimmunoassay ausgearbeitet. Antisera gegen Nortestosteron-17β-hemisuccinat- und gegen Nortestosteron-3-carboxymethyloxim-Rinderserumalbumin wurden an Ziegen gewonnen und für die Bestimmung in einem optimalen Gemisch verwendet. Die Kreuzreaktion mit endogenen Steroiden kann auf diese Weise unterdrückt werden, so daß die Methode als ein Screeningtest für Anabolika vom Nortestosterontyp im nativen sowie hydrolysierten Harn und im Plasma für den Zweck der Dopingkontrolle verwendet werden kann.

Introduction

We recently reported a radioimmunoassay of 17α-alkylated anabolic steroids suitable for doping control (1). Another large group of anabolics is related to 19-nortestosterone. This includes parenterally active nortestosterone and its 17β-hydroxyesters, as well as orally active 17α-alkyl-19-norsteroids.

The radioimmunological methods so far described for the detection of these compounds, use rabbit (2) or sheep (3) antisera raised against nortestosterone-3-carboxymethyloxime-bovine serum albumin conjugate. They suffer from unsatisfactory specifity, owing to the limited structural differences between nortestosterone and natural androgens. In order to enhance the selectivity of steroid radioimmunoassay, the use of the mixture of antisera obtained with two different immunogens has been suggested (4, 5). This approach has been applied in the present work.

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Materials and Methods

Materials

[1,2,4(n)^{3}H]Nortestosterone (17β-hydroxy-19-nor-4-androsten-3-one), specific radioactivity 1514 GBq · mmol^{-1} from Radiochemical Centre, Amersham, England was purified by paper chromatography in the system cyclohexane/toluene/methanol/water (volumes 9 + 1 + 8 + 2). Nortestosterone 17β-hemisuccinate and 3-carboxymethyloxime were prepared according to *Erlanger* et al. (6). The identity of nortestosterone derivatives was confirmed by ultraviolet and infrared spectroscopy as described elsewhere (7). The conjugates with bovine serum albumin were prepared by the mixed anhydride method (6) with minor modifications as described earlier (1). The final steroid–albumin molar ratios in conjugates assessed from ultraviolet spectra were 23 and 21 for nortestosterone 17β-hemisuccinate and 3-carboxymethyloxime, respectively. Anabolic steroids were obtained from Schering A.G. (Berlin), Spofa (Czechoslovakia) and Ciba (Switzerland). Other non-radioactive steroids were purchased from Koch-Light (England). *Helix pomatia* digestive juice, prepared in our laboratory and containing a minimum 50 000 I.U./ml β-glucuronidase, was used for enzymatic hydrolysis of urine. Chemicals were of analytical grade; bidistilled solvents and silanized glassware were used for radioimmunoassay.

Methods

Preparation of antisera

Goats were immunized with each conjugate according to the immunization schedule described previously (1). The highest titre was achieved only 8 weeks after the first immunization with 17β-conjugate, and after 12 weeks for the immunization with the 3-conjugate. Goats were then killed and bled out, and the sera were lyophilized. The stock solution of combined antisera was prepared by mixing 125 µl of anti-nor-testosterone-17β-hemisuccinate-bovine serum albumin with 250 µl of anti-nor-testosterone-3-carboxymethyl-oxime-bovine serum albumin, and dilution of the resulting mixture with 4.625 ml of buffer (see further). Combined antisera were divided into 100 µl portions and kept frozen at −15 °C. Before use they were further diluted 50-fold with buffer.

Sample preparation

Plasma or untreated urine were processed as follows: Plasma (100 µl) or urine (5 ml) was extracted with diethyl ether (3 ml and 5 ml, respectively), the organic phase was washed with saline (9 g · l^{-1}) and decanted into reaction tubes. The extraction tubes were rinsed with ether (1 ml) which was added to the main portion, and the solvent was evaporated.

When conjugated steroids were to be analyzed, the following procedure was applied: Urine (0.5 ml), 0.1 mol · l^{-1} sodium acetate buffer, pH 4.5 (0.5 ml) and β-glucuronidase solution in buffer (100 µl, at least 5000 I.U.) were incubated overnight at 37 °C. After cooling, the mixture was extracted with diethyl ether (5 ml). The ether extract was washed with 0.1 mol · l^{-1} NaOH in saline and then saline only (1 ml each) and an aliquot of the ether extract (1 ml, equivalent to 100 µl of urine) was transferred into reaction tubes and evaporated.
Simultaneously, a calibration curve of standard nortestosterone (usually 0, 0.2, 0.5, 1, 2, 4 and 8 ng) was worked up in the same way.

Radioimmunoassay

[^{3}H]Nortestosterone (250 Bq) and appropriately diluted mixture (see results, optimal dilution) of antisera in buffer (100 µl each) were added to dry residues of samples or standards, and the volume was adjusted to 0.5 ml with buffer. 0.1 mol · l^{-1} sodium phosphate, pH 7.2 containing 9 g · l^{-1} saline, 1.0 g · l^{-1} sodium azide and 1.0 g · l^{-1} bovine serum albumin was used. The samples were shaken on a Vortex mixer, incubated at 37 °C with shaking for 30 min, then left at 4 °C for 1 hour. A cold, stirred suspension of dextran-coated charcoal (0.5 ml of 2.5 g · l^{-1} Norit A and 0.25 g · l^{-1} Dextran 70) was added to each sample, the tubes were shaken briefly on a Vortex mixer, then left at 4 °C for 10 min. Following 10 min centrifugation at 2500 rpm an aliquot of supernatant (0.5 ml) was removed for determination of radioactivity. Radioactivity was measured on a Betaszint BF 5000 liquid scintillation spectrometer as described previously (1).

Determination of binding characteristics of antisera

The titre was expressed as the highest final dilution of serum at which more than 50% of given amount of labelled steroid (250 Bq) remained in the supernatant under the conditions of the assay. Apparent association constants were calculated from saturation curves obtained with nortestosterone as a ligand using a *Scatchard* plot corrected for non-specific binding (8). The specifity was evaluated from the ability of steroid competitors to cross-react with antibodies and their mixture. From the percentage of radioactivity remaining in the supernatant in the presence of competitor and respective amounts of the steroids, the log – logit straight lines were constructed (9), each from at least five points. Hence, the amounts of steroids required for 50% displacement of tracer were determined.

Results

Characterization of antibodies

The highest antibody titres obtained from goats immunized with nortestosterone-17β-hemisuccinate- and nortestosterone-3-carboxymethyloxime-bovine serum albumin conjugates were 1:32000 and 1:8000, respectively. The corresponding association constants with nortestosterone as a ligand at 25 °C were 1.31×10^9 l · mol^{-1} and 1.78×10^9 l · mol^{-1} respectively.

Cross-reactivities of 24 steroids with each antiserum are shown in the first two columns of table 1. Three nortestosterone 17β-hydroxyesters, two 5α-reduced metabolites, norethandrolone and four other orally active anabolic steroids, as well as several hormonal steroids (especially androgens) and their metabolites, were tested. As can be seen, both antibodies react with 19-norsteroids, but to different extents. The antibody raised against the 17β-conjugate recognized preferably those steroids possessing the Δ^4-3-oxogrouping (nortestosterone 17β-hydroxyesters, testosterone, androstenedione, progesterone). Besides the hydrogen atom on C_{10} ("19-nor" group), a structural determinant for antibody against 3-conjugate appeared to be the 17β-hydroxy group, while the stereochemistry of the A-ring was less important; 17β-hydroxy-19-norsteroids, estradiol, testosterone and 17β-androstanediols were the most effective competitors.

Determination of the optimal ratio of antisera in the mixture

To minimize cross reactions of naturally occurring steroids and, at the same time, to retain the ability to bind 19-nor-compounds, the optimal composition of the mixture of both antisera was established according to *Rodbard* (5). 4-Androstene-3,17-dione and 5α-androstane-3α, 17β-diol were chosen as model compounds: the former cross-reacted considerably only with antibody against the 17β-conjugate (Ab. 1), whereas the latter showed high cross-reaction with antibody raised against the 3-oxime conjugate (Ab. 2) only.

Various volumes of both antisera, each diluted so that a given amount of nortestosterone (1 ng) caused 50% displacement of tracer (1:12000 for Ab. 1 and 1:4000 for Ab. 2, respectively), were combined to obtain the following mixtures: 1.0 Ab. 1; 0.7 Ab. 1 + 0.3 Ab. 2; 0.5 Ab. 1 + 0.5 Ab. 2; 0.3 Ab. 1 + 0.7 Ab. 2; 1.0 Ab. 2. The amounts of nortestosterone and both competitors required for 50% displacement of radioactive tracer ("ID_{50} and ED_{50}", respectively, according to *Rodbard* (5)) were then established with each mixture. The ratios ED_{50}/ID_{50} for both model compounds were calculated and plotted against relative composition of the antisera-mixture. As shown on figure 1, two straight lines were obtained, their intercept giving the optimal composition of the mixture. The value found was 0.60 Ab. 1 and 0.40 Ab. 2. It corresponded to two volumes of undiluted Ab. 2 and one of Ab. 1, further diluted to the working dilution as described in methods.

The cross reactivities of the steroids under study with the optimal antibody mixture are shown in the third column of table 1.

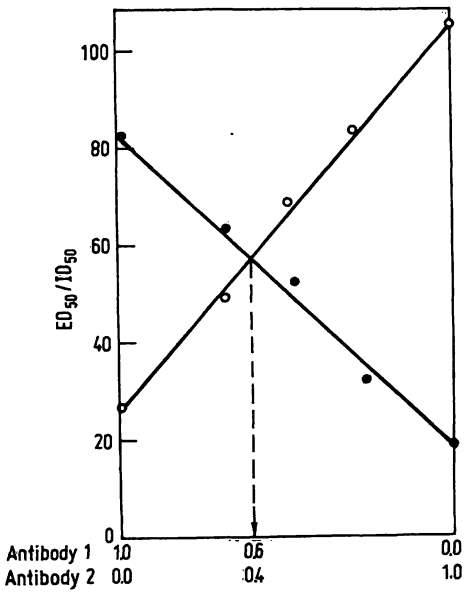

Fig. 1. Determination of the optimal ratio of antisera in the mixture. Ratios ED_{50}/ID_{50} (where ED_{50} means the amount of the competitor and ID_{50} amount of nortestosterone, respectively, required for 50% displacement of radioactive tracer) were plotted against the relative composition of antisera-mixture. Open circles: ED_{50} of androstenedione/ID_{50}, full circles: ED_{50} of 5α-androstane-3α, 17β-diol/ID_{50}.

Antibody 1: Antiserum with antibodies against nortestosterone-17β-hemisuccinate-bovine serum albumin conjugate

Antibody 2: Antiserum with antibodies against nortestosterone-3-carboxymethyloxime-bovine serum albumin conjugate

Tab. 1. Cross-reactivities of various steroids with goat nortestosterone antibodies and their mixtures.

Steroid (systematic name)	Cross-reaction in %		
	Ab. 1[a]	Ab. 2[b]	Ab. 1 + Ab. 2 0.6 0.4
Nortestosterone and its analogues			
Nortestosterone (17β-hydroxy-19-nor-4-androsten-3-one)	100	100	100
Nortestosterone phenylpropionate	10.1	1.73	5.02
Nortestosterone acetate	30.2	2.81	19.2
Nortestosterone hemisuccinate	18.9	2.50	10.5
Nordihydrotestosterone (17β-hydroxy-19-nor-5α-androstan-3-one)	21.4	80.7	27.4
5α-androstane-19-nor-3, 17β-diol	0.46	20.4	8.26
Norethandrolone (17α-ethyl-17β-hydroxy-19-nor-4-androsten-3-one)	37.6	5.40	29.9
Other anabolic steroids			
Drostanolone (2 α-methyl-17β-hydroxy-5α-androstan-3-one)	0.25	1.98	0.67
Methandienone (17α-methyl-17β-hydroxy-1,4-androstadien-3-one)	1.09	0.60	0.97
Methenolone (1-methyl-17β-hydroxy-5α-androst-1-en-3-one)	5.30	0.30	1.74
Stanazolol (17α-methyl-17β-hydroxy-5α-androstane-3,2-C-pyrazol)	< 0.1	0.12	< 0.1
Androgens and their metabolites			
Testosterone	7.70	6.50	6.90
Dihydrotestosterone (17β-hydroxy-5α-androstan-3-one)	2.06	3.12	2.59
Androstenedione (4-androstene-3,17-dione)	3.86	0.93	1.83
Androsterone (3α-hydroxy-5α-androstan-17-one)	0.30	< 0.1	0.11
Dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one)	0.40	< 0.1	0.15
Etiocholanolone (3α-hydroxy-5β-androstan-17-one)	0.20	< 0.1	< 0.1
5α-androstane-3α, 17β-diol	0.90	6.00	1.01
5β-androstane-3α, 17β-diol	0.45	4.01	0.76
Epitestosterone (17α-hydroxy-4-androsten-3-one)	1.98	1.92	1.94
Other hormonal steroids			
Cortisol	< 0.1	< 0.1	< 0.1
Estradiol	0.38	7.50	0.79
Progesterone	2.13	0.56	1.32
Ethinylestradiol (17α-ethinyl-1,3,5 (10) estratriene-3, 17β-diol[c]	< 0.1	< 0.1	< 0.1

[a] goat anti-nortestosterone-17β-hemisuccinate bovine serum albumin

[b] goat anti-nortestosterone-3-carboxymethyloxime bovine serum albumin

[c] synthetic estrogen

Reliability criteria of the method

Accuracy was expressed as recovery of nortestosterone added to urine and/or plasma samples processed as described under methods. For each group representing six analyses the respective coefficients of variation were also calculated (precision). The results for untreated and hydrolyzed urine are summarized in table 2, for plasma in table 3.

Sensitivity was determined according to *Ekins* (10), as the lowest amounts of nortestosterone added to urine or plasma samples which could be detected with 95% probability. The respective values for untreated urine, hydrolyzed urine and plasma were 287, 329 and 22 pg.

Discussion

Compared with the radioimmunoassay of 17α-alkylated anabolic steroids, the detection of nortestosterone and its metabolites presents further problems. These arise from similarities in structure and metabolism between nortestosterone and natural androgens, namely testosterone (11).

When nortestosterone 3-carboxymethyloxime conjugate only is used as an immunogen, considerable cross-reaction of various 17β-hydroxysteroids occurs, whereas detection of 19-norcompounds without a free 17β-hydroxy group (nortestosterone esters, 17-oxo-19-norsteroids) is limited.

In this work, the aim was to increase group selectivity as needed in screening methods in doping control for anabolics. Using the mixture of antisera elicited by two different immunogens the undesired cross-reaction of several steroids can be reduced without considerable loss of ability to detect nortestosterone analogues. The only hormone, the cross-reaction of which cannot be overcome, is testosterone.

The reliability criteria of the method described are comparable with common radioimmunoassay procedures. It was applied successfully in doping control for anabolics at the European athletics championship, Prague 1978. Both unprocessed and hydrolyzed urine extracts were used in the assay. 5–6 Control urine samples were analyzed within each series. Those values which were higher by 2 S.D. from the mean of controls were considered as positive.

Tab. 3. Accuracy and precision of nortestosterone radioimmunoassay in plasma. Increasing amounts of nortestosterone were added to normal male plasma, which was processed as described in methods. Six determinations were carried out in each group.

Nortestosterone added	found mean ± S.D. pg	Coefficient of variation %	Recovery %
none	120 ± 10.9	9.8	–
50	172 ± 16.0	9.30	101.2
100	209 ± 18.3	8.76	95.0
200	284 ± 28.5	10.04	88.8
	mean:	9.30	95.0

Tab. 2. Accuracy and precision of nortestosterone radioimmunoassay in urine. Increasing amounts of nortestosterone were added to normal male urine, which was further processed as described in methods. Untreated and/or enzymatically hydrolyzed urine samples were analyzed, with six determinations in each group.

Analyzed material: Untreated urine (5 ml)				Hydrolyzed urine (100 µl)		
Nortestosterone added pg	found mean ± S.D. pg	Coefficient of variation %	Recovery %	Nortestosterone found mean ± S.D. pg	Coefficient of variation %	Recovery %
none	1820 ± 105	5.77	–	2780 ± 249	8.96	–
100	1950 ± 101	5.20	101.6	2849 ± 200	7.02	98.9
200	2095 ± 144	6.87	103.7	3029 ± 182	6.01	101.6
400	2111 ± 146	6.92	95.1	3265 ± 186	5.70	102.7
		mean: 6.19	100.1		mean: 6.92	101.1

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